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*Published in:*  
Vaccine

*Link to article, DOI:*  
[10.1016/j.vaccine.2017.11.059](https://doi.org/10.1016/j.vaccine.2017.11.059)

*Publication date:*  
2018

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*  
Kristensen, C. S., Kvisgaard, L. K., Pawlowski, M., Holmgaard Carlsen, S., Hjulsager, C. K., Heegaard, P. M. H., Bøtner, A., Stadejek, T., Haugegaard, S., & Larsen, L. E. (2018). Efficacy and safety of simultaneous vaccination with two modified live virus vaccines against porcine reproductive and respiratory syndrome virus types 1 and 2 in pigs. *Vaccine*, 36(2), 227-236. <https://doi.org/10.1016/j.vaccine.2017.11.059>

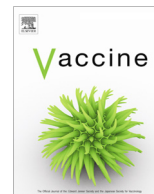
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# Efficacy and safety of simultaneous vaccination with two modified live virus vaccines against porcine reproductive and respiratory syndrome virus types 1 and 2 in pigs

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## ARTICLE INFO

### Article history:

Received 24 July 2017

Received in revised form 17 November 2017

Accepted 18 November 2017

Available online 27 November 2017

### Keywords:

Porcine reproductive and respiratory syndrome virus

Vaccination

Swine

PRRSV

## ABSTRACT

The objective of the study was to compare responses of pigs vaccinated with a PRRS MLV vaccine against PRRSV-1 or PRRSV-2 with the responses of pigs vaccinated simultaneously with both vaccines. Furthermore, the efficacy of the two PRRSV MLV vaccination strategies was assessed following challenge. The experimental design included four groups of 4-weeks old SPF-pigs. On day 0 (DPV0), groups 1–3 (N = 18 per group) were vaccinated with modified live virus vaccines (MLV) containing PRRSV-1 virus (VAC-T1), PRRSV-2 virus (VAC-T2) or both (VAC-T1T2). One group was left unvaccinated (N = 12). On DPV 62, the pigs from groups 1–4 were mingled in new groups and challenged (DPC 0) with PRRSV-1, subtype 1, PRRSV-1, subtype 2 or PRRSV-2. On DPC 13/14 all pigs were necropsied. Samples were collected after vaccination and challenge. PRRSV was detected in all vaccinated pigs and the majority of the pigs were positive until DPV 28, but few of the pigs were still viremic 62 days after vaccination. Virus was detected in nasal swabs until DPV 7–14. No overt clinical signs were observed after challenge. PRRSV-2 vaccination resulted in a clear reduction in viral load in serum after PRRSV-2 challenge, whereas there was limited effect on the viral load in serum following challenge with the PRRSV-1 strains. Vaccination against PRRSV-1 had less impact on viremia following challenge. The protective effects of simultaneous vaccination with PRRSV Type 1 and 2 MLV vaccines and single PRRS MLV vaccination were comparable. None of the vaccines decreased the viral load in the lungs at necropsy. In conclusion, simultaneous vaccination with MLV vaccines containing PRRSV-1 and PRRSV-2 elicited responses comparable to single vaccination and the commercial PRRSV vaccines protected only partially against challenge with heterologous strains. Thus, simultaneous administration of the two vaccines is an option in herds with both PRRSV types.

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## 1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most devastating infections in most swine producing countries globally. In the US, the annual losses due to PRRS reach \$644 million annually [1] and the losses after an acute outbreak has been estimated to be between 59 and 379 Euro/sow in Holland [2].

Therefore, huge efforts are put into the elimination and control of the PRRS virus (PRRSV). Due to horizontal transmission of PRRSV [3], the risk of PRRSV infection is high in swine dense areas and therefore the strategy employed in most farms in Denmark and other parts of Europe is to establish a PRRS stable sow herd where sows are PRRSV antibody positive and PRRS virus negative and wean PRRSV free pigs. PRRSV vaccines are commonly used to immunize young breeding animals before introduction to the sow herd.

Both Modified Live Virus (MLV) vaccines and killed vaccines are available, but the efficacy of killed PRRS vaccines in stimulating protective immunity is questionable [4] and therefore MLV vaccines are used in most herds. Several studies have shown good

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**Table 1**

Experimental design. The pigs were allocated to three vaccination groups vaccinated with MLV vaccines containing PRRSV-1 (VAC-T1), PRRSV-2 (VAC-T2) or both (VAC-T1T2). Each of the vaccination groups were split into three different groups prior to challenge. The NON-VAC group was kept as unvaccinated control group. Four pigs died prior to challenge and one pig was excluded due to lack of seroconversion after vaccination resulting in a lower number of pigs in some of the challenge groups (4 or 5 pigs per group instead of 6 pigs).

| Group    | No. pigs        | PRRSV vaccination                        | PRRSV challenge   |
|----------|-----------------|--|---|
| VAC-T1   | 18 <sup>a</sup> | Porcilis®PRRS VET                        | 5 <sup>a</sup> pigs PRRSV-1 subtype 1<br>5 <sup>a</sup> pigs PRRSV-2<br>5 <sup>a</sup> pigs PRRSV-1 subtype 2 |
| VAC-T2   | 18              | Ingelvac®PRRS VET                        | 6 pigs PRRSV-1 subtype 1<br>6 pigs PRRSV-2<br>6 pigs PRRSV-1 subtype 2  |
| VAC-T1T2 | 18 <sup>a</sup> | Porcilis®PRRS VET +<br>Ingelvac®PRRS VET | 6 pigs PRRSV-1 subtype 1<br>6 pigs PRRSV-2<br>4 <sup>a</sup> pigs PRRSV-1 subtype 2                           |
| NON-VAC  | 12              | No vaccination                           | 4 pigs PRRSV-1 subtype 1<br>4 pigs PRRSV-2<br>4 pigs PRRSV-1 subtype 2  |

efficiency of MLV against challenge with related strains [5]. Some studies also found partial protection against challenge with more divergent strains, whereas others found a poor cross-protection of vaccines containing more divergent strains [reviewed in 5]. These apparent differences in outcome of different experiments are probably due to the different experimental designs, different vaccines used, different challenge strains, different breeds, age of the animals, challenge dose etc. Nevertheless, it is generally accepted that the degree of protection elicited by PRRSV vaccines are related to the level of genetic and antigenic similarity between the challenge and vaccine strain, even though that the level of genetic and antigenic similarity is not necessarily predictive of protection [6].

Both PRRSV-1, subtype 1 and PRRSV-2 are circulating and causing disease in some European countries [7,8]. In contrast, PRRSV-1 strains belonging to subtypes 2, 3, and 4 have never been detected in Western Europe [23]. In Denmark, it is common that pigs are simultaneously vaccinated with two different PRRS MLV vaccines containing PRRSV-1 and PRRSV-2. There is limited published data on the impact on duration of viremia, immune responses and efficacy after administration of two PRRS MLV vaccines containing PRRSV-1 and PRRSV-2 at the same time [9].

The objective of the study was therefore to compare the safety and efficacy of single PRRS MLV vaccinated pigs with responses in pigs simultaneous vaccinated with PRRSV Type 1 and 2 vaccines.

## 2. Materials and methods

### 2.1. Experimental design

In total, 66 four-week-old PRRSV-negative pigs were included in the study. The pigs were purchased from a specific pathogen-free herd and tested free of a range of pathogens including PRRSV, swine influenza A virus, *Actinobacillus pleuropneumoniae* (AP) and *Mycoplasma hyopneumoniae* by serology prior to the study. The pigs also tested negative by real-time PCR for Porcine circovirus type 2 (PCV2) virus at arrival. The pigs were housed at the experimental animal facilities at the National Veterinary Institute under appropriate biosecurity conditions. On arrival, the pigs were randomly allocated into four groups housed in separate rooms.

One week after arrival (0 days post vaccination, DPV 0), the pigs in groups 1–3 (N = 16) were vaccinated with either Porcilis® PRRS VET (MSD Animal Health, Denmark) containing PRRSV-1 (VAC-T1), Ingelvac® PRRS VET (Boehringer Ingelheim Animal Health, Denmark) containing PRRSV-2 (VAC-T2) or both vaccines simultaneously (VAC-T1T2) (Table 1). Porcilis® PRRS VET was

administered with 2 mL at the left side of the neck and Ingelvac® PRRS VET administered with 2 mL at the right side of the neck.

Nine weeks after vaccination (DPV 62), all pigs were moved to new separated groups according to the PRRSV strain they were planned to be challenged with (Table 1). The challenge was done with either PRRSV-1, subtype 1 (strain 18794 [10]), PRRSV-2 (strain 19407b) or PRRSV-1 subtype 2 (strain ILI6 [10]) according to Table 1. The PRRS-19407B had been isolated in January 1997 from the lungs of a stillborn pig. This pig originated from a swine herd with a sudden high occurrence of stillborn pigs and increased piglet mortality in the nursing period, consistent with an acute outbreak of PRRS. The following day (0 days post challenge (DPC 0), corresponding to DPV 63), all pigs were inoculated intranasally by placing the pigs on their buttocks perpendicular to the floor and expanding the neck fully. The inoculum was slowly dripped into the nostrils (2 mL/nostril) of the pigs taking approximately 3–5 min/pig.

The PRRSV-1, subtype 1 inoculum contained  $5 \times 10^5$  culture infective dose (TCID<sub>50</sub>/mL) of PRRSV (passage 6, PAM, 1 mL virus suspension in 3 mL MEM). The PRRSV-2 inoculum contained  $5 \times 10^5$  TCID<sub>50</sub>/mL of PRRSV (passage 3, Marc-145, 1 mL virus suspension in 3 mL MEM) and the PRRSV-1, subtype 2 inoculum contained  $3.7 \times 10^5$  TCID<sub>50</sub>/mL.

The study was carried out in accordance and permission granted by the Danish legislation on animal experiments (LBK nr 1306 – 23/11/2007; permission number 2014–15–0201–00091) and EU regulations on the use of laboratory animals for research.

### 2.2. Sampling

Blood samples were collected on days 2, 6, 14, 21, 28, 35, 42, 49, 56 and 62 DPV from vaccinated pigs and on day 62 for non-vaccinated control pigs (NON-VAC). Blood samples were also collected on days 1, 3, 5, 9 and 13 after challenge (DPC). Serum was separated from the blood and stored at –80 °C until test. Nasal swabs were collected on DPV 1, 2, 5, 14, and 21 and DPC 1, 3, 4, 5, and 9. The swabs were collected in 1 mL PBS and stored at –80 °C until test.

### 2.3. Clinical observation

A clinical score was assessed daily based on general health condition (normal, mild lethargic, lethargic or apathetic), respiration (normal, increased respiration, respiratory distress, severe respiratory distress), and appetite (normal, slow eating, not eating). Rectal

temperature was recorded on days –1, 0, 2, 5, and 6 DPV and 1, 2, 3, 4, 5, and 6 DPC.

#### 2.4. Necropsy

Of practical reasons half of the animals in each group was necropsied on each day on DPCs 13 and 14, respectively. The pigs were euthanized using a captive bolt immediately followed by a cut of *A. axillaris*. A gross pathological examination was performed. Lung pieces from the right and left caudal lobes and the right apical lobe were collected from all pigs and pooled in the same tube. From lungs with macroscopic lesions, a tissue section was collected from the affected area and stored in a separate tube. The tissue samples were stored at –80 °C until use.

#### 2.5. Laboratory methods

##### 2.5.1. RNA extraction and RT-qPCR assays

Total RNA was extracted from serum, nasal swabs and lung samples as previously described [10]. Known PRRSV positive samples and negative controls were included for at least each batch of 10 samples. Extracted RNA was stored at –80 °C.

A recent designed assay was used for the real-time RT-qPCR analyses [10]. The assay was designed specifically to detect PRRSV-1 subtype 1 and subtype 2 strains used in this study. The primers and probe target ORF2. For detection of PRRSV-2, the previously published RT-qPCR “Kleiboeker mod-1” primers and probe targeting ORF7 3'UTR were used [11].

##### 2.5.2. Conventional PCR and sequencing

The conventional PCR amplifications of ORF5 and ORF7 and the sequencing were performed as previously described for PRRSV-2 [8]. For the sequencing of PRRSV-1, subtype 1 and subtype 2, amplification of ORF5-ORF6 was performed using a ORF5-fw and a ORF6-Rev primer [12] resulting in a PCR fragment of 1288 bp. PCR and sequencing primers were the same. Only ORF5 sequences were included in the analysis. The sequencing of PRRSV-1 ORF7 for confirmation of some RT-PCR results was performed as earlier described [7].

##### 2.5.3. C-reactive protein (CRP)

Acute phase proteins (APPs) are induced in response to infection and because measurable clinical signs are difficult to induce in pigs with PRRSV, measurement of the porcine APP CRP was included as an “objective” measure of infection. CRP was analyzed by a sandwich type ELISA using dendrimer-coupled cytidine diphosphocholine (a CRP-binding ligand) in the coating layer as earlier described employing polyclonal rabbit anti-human antibodies with cross-reactivity towards porcine CRP followed by peroxidase-conjugated goat anti rabbit antibody for detection (both antibodies from DAKO, Glostrup, Denmark) [13]. The lower limit of quantification of the assay was 780 ng/ml.

#### 2.6. Laboratory analyses

All serum and nasal swab samples collected at 2, 6, 14, 21, 28, 35, 42, and 62 DPV from vaccinated pigs were tested in real-time RT-PCR. Samples from VAC-T1 and VAC-T1T2 pigs were tested in the real-time RT-PCR for PRRSV-1 and samples from VAC-T2 and VAC-T1T2 pigs in the real-time RT-PCR specific for PRRSV-2.

Samples from VAC-T1 and VAC-T2 pigs, that were negative at 42 DPV in real-time RT-PCR, were not tested day 49 and 56. Samples from VAC-T1T2 pigs negative for both PRRSV-1 and PRRSV-2 at 42 DPV in real-time RT-PCR, were not tested DPV 49 and 56. All other samples from VAC-T1, VAC-T2 and VAC-T1T2 were tested DPV 49 and 56.

From samples positive in real-time RT-PCR at DPV 62, a new RNA extraction was performed from the original sample and tested again in real-time RT-PCR. If the real-time RT-PCR was positive, a conventional PCR targeting ORF7 was performed and the PCR product confirmed by sequencing. All samples from NON-VAC pigs were tested in real-time RT-PCR for both PRRSV-1 and PRRSV-2 at DPC-1.

Following challenge, nasal swabs and serum collected 1, 3, 5, 9, and 13 DPC were tested in real-time RT-PCR specific for the challenge strain. Serum from the same days was tested for CRP.

The three lung pieces collected from each pig at necropsy were pooled and tested by real-time RT-PCRs specific for the challenge strain and the PRRSV virus species used for vaccination. ORF5 of the virus RNA detected in the lungs of one pig from each group was sequenced as described above.

#### 2.7. Statistical analysis

The binary response of PCR positive pigs after vaccination were analyzed separately, for PCR PRRSV-1 (VAC-T1 against VAC-T1T2) and for PCR PRRSV-2 (VAC-T2 against VAC-T1T2). The analyses were performed using the GLIMMIX procedure with logit as link function and including vaccine and time as a fixed effect and with the repeated measurement on the same pig during time, having a AR(1) covariance structure. The statistical package SAS version 9.2 (SAS institute Inc., Cary, NC, USA) was used. A value of  $P < .05$  was considered significant. Group sample sizes of 18 and 18 achieve 81% power to detect an odds ratio of 0.286 in a design with 9 repeated measurements having a AR(1) covariance structure when the proportion from group 2 is 0.600, the correlation between observations on the same subject is 0.680, and the alpha level is 0.050.

### 3. Results

#### 3.1. Limited clinical signs observed after vaccination and challenge

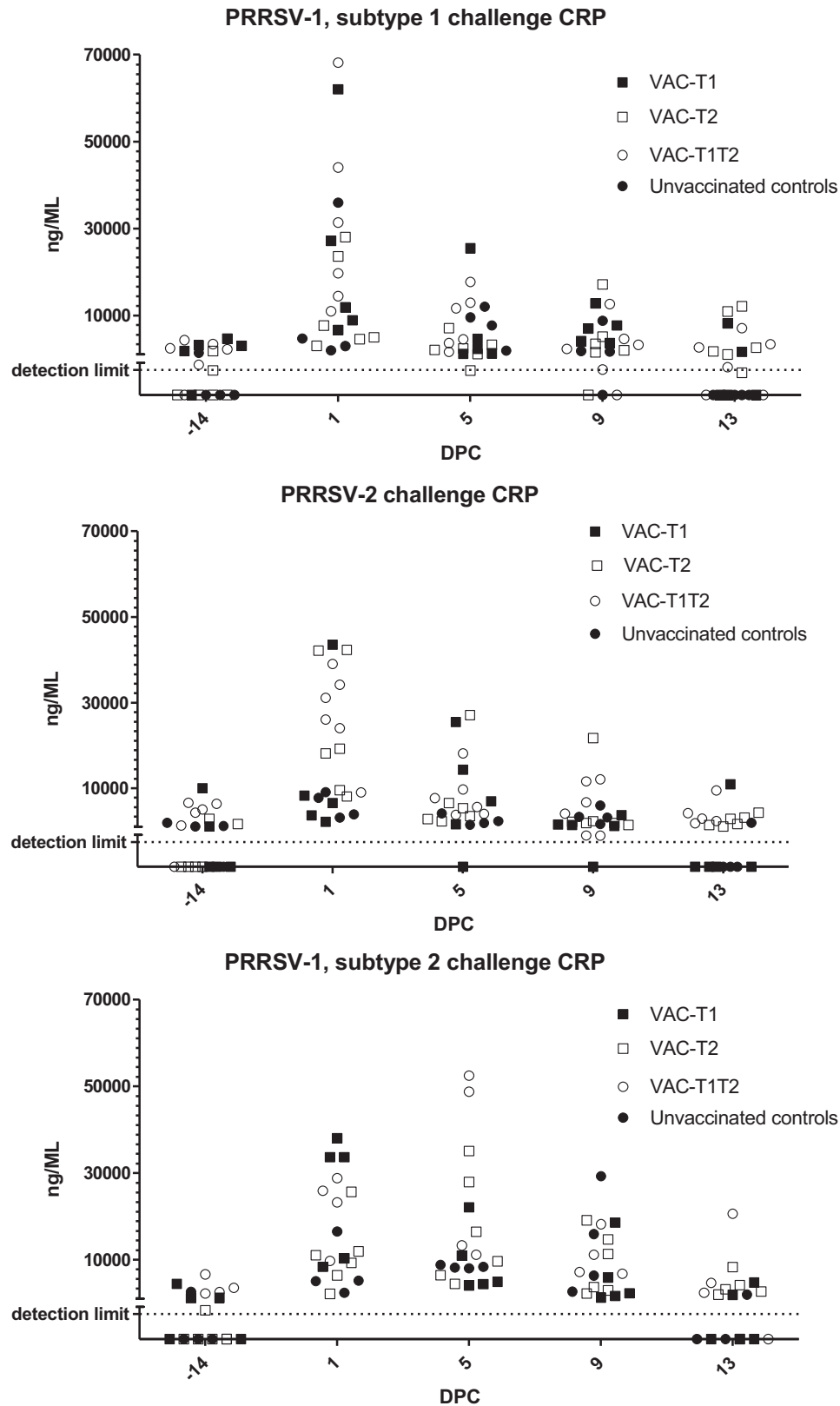
No respiratory clinical symptoms were observed after vaccination. Four pigs were suffering from diarrhea and were euthanized due to poor body condition (three VAC-T1 pigs at 14, 23, and 23 DPV, one VAC-T1T2 pig at 37 DPV).

Several of the vaccinated pigs that were challenged with PRRSV-1, subtype 2, got a clinical score at DPC 5; one VAC-T1T2 pig was lethargic and was not eating; two VAC-T1 pigs, three VAC-T2 pigs and one VAC-T1T2 pig were mildly lethargic. None of the pigs challenged with PRRSV-1, subtype 1 or PRRSV-2 developed clinical signs after challenge. Some animals experienced a slight increase in rectal temperature at DPC 3, however none of the pigs developed fever as defined as rectal temperature above 40.5 °C (data not shown).

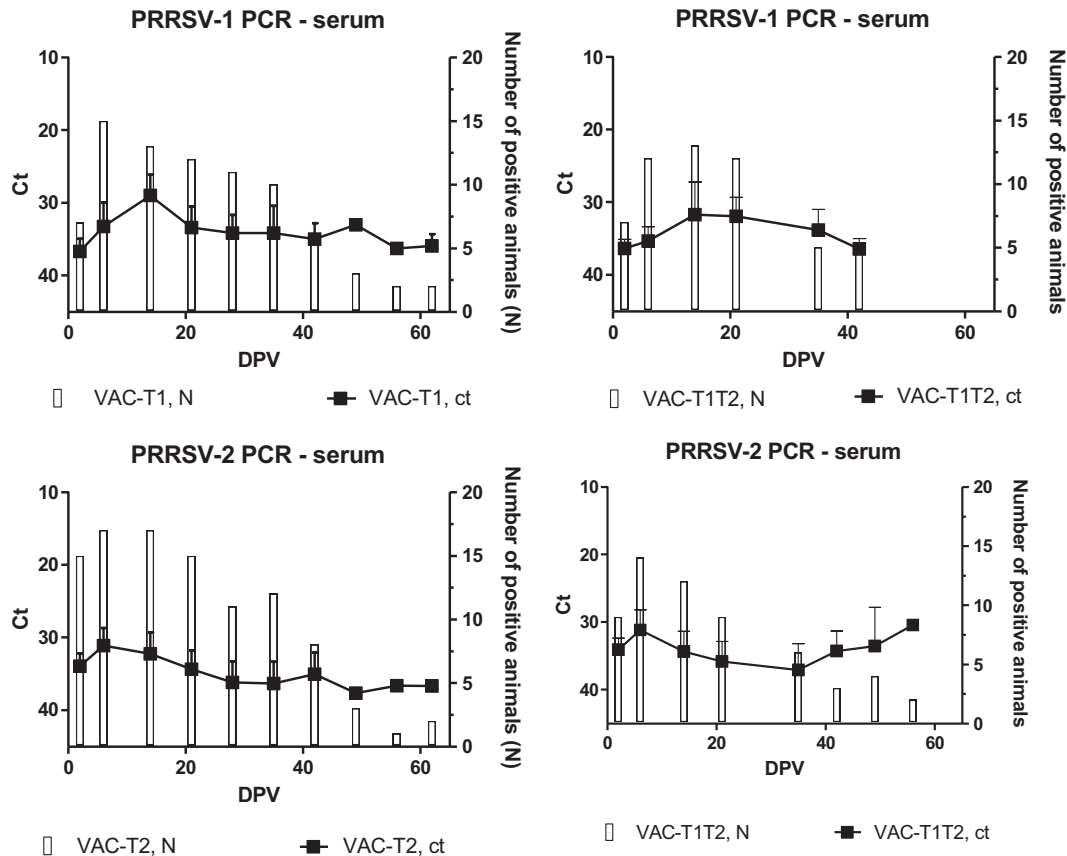
#### 3.2. Acute phase protein responses were induced in response to challenge

All pre-challenge serum concentrations of CRP were below 10 µg/ml which generally is regarded as the upper cut-off for healthy animals [14] (Fig. 1). After challenge, PRRSV-1, subtype 1 and PRRSV-2 induced similar patterns of elevated CRP concentrations peaking at 1 DPC while the PRRSV-1, subtype 2 strain induced a slower and more protracted response with elevated CRP concentrations found at 1 and 5 DPC and, although less pronounced, at 9 DPC.

The CRP responses of the different vaccinated groups and the unvaccinated controls were striking in that the group vaccinated with both vaccines consistently had the highest number of samples



**Fig. 1.** C-reactive protein (CRP) responses to PRRSV challenge in vaccinated and unvaccinated pigs. Level of CRP (ng/mL) were measured in animals challenge with a PRRSV-1, subtype 1 strain (top), a PRRSV-2 strain (center) or a PRRSV-1, subtype 2 strain (bottom) at day -14, 1, 5, 9, and 13 after challenge (DPC). Groups of pigs were vaccinated with MLV against PRRSV-1 (VAC-T1), PRRSV-2 (VAC-T2) or both (VAC-T1T2) 63 days prior to challenge.



**Fig. 2.** Viral load expressed as mean Ct values and the number of positive pigs (N) in serum after vaccination with MLV against PRRSV-1 (VAC-T1), PRRSV-2 (VAC-T2) or both (VAC-T1T2). The serum samples were tested with an RT-PCR assay specific for PRRSV-1 (top) or PRRSV-2 (bottom). Samples with Ct values above 40 were considered negative. DPV = days post vaccination.

with CRP concentrations above 10 µg/ml, followed by the single vaccinated groups and with the unvaccinated group consistently having the fewest samples above the cut-off (Fig. 1).

### 3.3. PRRSV was detected for a prolonged time in serum following vaccination

One VAC-T1T2 pig did not develop viremia after vaccination, either because this animal was mistakenly not vaccinated or that the pigs were unable to respond to vaccination. Based on this, the pig was excluded from further analysis. The blood samples from two VAC-T1 and all VAC-T1T2 pigs collected at 28 DPV were accidentally lost after sampling and prior to analysis. Apart from the pig mentioned above, all vaccinated pigs became viremic at least one day following vaccination as measured by real-time RT-PCR for PRRSV-1 and/or PRRSV-2 according to the vaccine used (Fig. 2).

Following vaccination, the VAC-T1 and VAC-T1T2 pigs had the lowest mean Ct value in serum. None of the VAC-T1T2 pigs were positive after DPV 42, whereas two VAC-T1 pigs remained weakly positive at 62 DPV (Fig. 2). Sequencing of ORF5 from these samples confirmed that they were vaccine virus positive. No significant differences were found comparing the proportion of positive pigs between the VAC-T1 and VAC-T1T2 groups of pigs ( $p = .21$ ), but the level of virus RNA in serum of VAC-T1T2 pigs tended to be higher than in the VAC-T1 pigs.

The majority of pigs vaccinated with the PRRSV-2 vaccine (VAC-T2 and VAC-T1T2) were positive for PRRSV-2 viral RNA in serum from DPV 6 to DPV 28 (Fig. 2), however, one VAC-T1T2 pig did not become positive until DPV 42. Eight VAC-T2 and three

VAC-T1T2 pigs tested positive also at DPV 42 and two VAC-T2 pigs were still weakly positive at DPV 62 (Fig. 2). Sequencing of ORF5 from these samples confirmed that they were PRRSV vaccine virus positive. Overall, the number of PRRSV-2 serum positive pigs was higher in the VAC-T2 group compared to the VAC-T1T2 group ( $p = .007$ ) (Fig. 2). Overall, the level of virus RNA in serum of VAC-T1T2 pigs was not different from the VAC-T2 pigs.

The highest proportion of nose swabs positive in real-time RT-PCR was seen at 5 DPV where 5–7 pigs in each group were positive (Table 2). At DPC 14, only one VAC-T1 and three VAC-T1T2 pigs tested positive for PRRSV-1, whereas none of the pigs were positive for PRRSV-2. At DPV 21 all nose swabs were negative (Table 2).

### 3.4. PRRSV were detected in sera and nasal swabs following challenge of vaccinated pigs

After challenge with PRRSV-1, subtype 1, all vaccinated pigs were positive in serum at minimum one time-point when tested by the real-time RT-PCR specific for PRRSV-1. All NON-VAC pigs were viremic at all sampling dates (Fig. 3). The level of virus in the NON-VAC group was in general higher than in the vaccinated groups. Overall, development of viremia was delayed and of shorter duration in the vaccinated pigs and all PRRSV-1 vaccinated pigs (VAC-T1 and VAC-T1T2) were negative in serum at DPC 13. Interestingly, two of the VAC-T2 pigs that tested negative at DPC 9 became positive again on DPC 13. To confirm these results the RT-PCR tests were repeated on new RNA extractions with similar results.

In nasal swabs, only two out of five pigs vaccinated and challenged with PRRSV-1, subtype 1 were positive and only at one



**Table 2**

Number of pigs found positive in nasal swabs when tested in real-time RT-PCR assays specific for either type 1 or type 2 PRRSV following vaccination (top) with PRRSV-1 (VAC-T1), PRRSV-2 (VAC-T2) or both (VAC-T1T2) and after challenge (bottom). DPV = days post vaccination. DPC = days post challenge. \*One VAC-T1T2 vaccinated pig was excluded from the study due to lack of seroconversion and three VAC-T1 and one VAC-T1T2 pig died prior to challenge.

| DPV | PRRSV-1 RT-PCR    |  |                     |  | PRRSV-2 RT-PCR   |  |                     |  |
|-----|-------------------|--|---------------------|--|------------------|--|---------------------|--|
|     | VAC-T1<br>N = 18* |  | VAC-T1T2<br>N = 17* |  | VAC-T2<br>N = 18 |  | VAC-T1T2<br>N = 17* |  |
| 1   | 0                 |  | 0                   |  | 1                |  | 0                   |  |
| 2   | 0                 |  | 2                   |  | 1                |  | 2                   |  |
| 5   | 5                 |  | 5                   |  | 7                |  | 5                   |  |
| 14  | 1                 |  | 3                   |  | 0                |  | 0                   |  |
| 21  | 0                 |  | 0                   |  | 0                |  | 0                   |  |

| Vaccine DPC | PRRSV 1-subtype 1 challenge |                 |                   |                    | PRRSV-2 challenge |                 |                   |                   | PRRSV 1-subtype 2 challenge |                 |                     |                   |
|-------------|-----------------------------|-----------------|-------------------|--------------------|-------------------|-----------------|-------------------|-------------------|-----------------------------|-----------------|---------------------|-------------------|
|             | VAC-T1<br>N = 5*            | VAC-T2<br>N = 6 | VAC-T1T2<br>N = 6 | Controls<br>N = 4* | VAC-T1<br>N = 5*  | VAC-T2<br>N = 6 | VAC-T1T2<br>N = 6 | Controls<br>N = 4 | VAC-T1<br>N = 5             | VAC-T2<br>N = 6 | VAC-T1 T2<br>N = 4* | Controls<br>N = 4 |
| 1           | 0                           | 0               | 0                 | 0                  | 0                 | 0               | 0                 | 1                 | 0                           | 2               | 0                   | 0                 |
| 3           | 0                           | 2               | 0                 | 2                  | 4                 | 0               | 0                 | 1                 | 1                           | 4               | 2                   | 2                 |
| 4           | 0                           | 3               | 4                 | 3                  | 4                 | 0               | 0                 | 1                 | 5                           | 6               | 3                   | 3                 |
| 5           | 0                           | 3               | 2                 | 3                  | 5                 | 0               | 1                 | 3                 | 4                           | 4               | 4                   | 4                 |
| 9           | 2                           | 0               | 0                 | 2                  | 0                 | 0               | 0                 | 0                 | 2                           | 2               | 2                   | 3                 |

sampling (DPC 9) whereas 4 and 2 pigs out of 6 double vaccinated pigs were positive at DPC 4 and 5, respectively. Out of the four unvaccinated pigs challenged with PRRSV-1, subtype 1, two-three were positive in nasal swabs at DPC 3–9, which were similar to the pigs vaccinated with PRRSV-2 apart from no positive pigs were seen in this group at DPC 9 (Table 2).

The challenge with PRRSV-2 resulted in only one positive VAC-T2 pig and one positive VAC-T1T2 pig in serum the day after challenge (DPC 1) (Fig. 3). At all other samplings, all VAC-T2 and VAC-T1T2 pigs tested negative for PRRSV-2. All NON-VAC pigs were positive for Type 2 PRRSV at all sampling days. The VAC-T1 pigs were positive for PRRSV-2 at DPC 1, 3, and 5, became negative at DPC 9 and then became positive again at DPC 13 (Fig. 3). To confirm these results the real-time RT-PCR tests were repeated on new RNA extraction with similar results.

Of the 12 pigs vaccinated and challenged with PRRSV-2, only one pig was positive in nasal swabs (at DPC 5) (Table 2). In the unvaccinated control group, only 1 of 4 pigs was positive at DPCs 1, 3, and 4, whereas 3 were positive at DPC 5. Interestingly, nasal shedding of PRRSV were more pronounced in pigs vaccinated with a vaccine containing PRRSV-1 in that 4–5 of the pigs were positive at DPC 3–5 (Table 2).

Following challenge with PRRSV-1, subtype 2, all pigs apart from two pigs in the VAC-T1T2 group were viremic at DPVs 3, 5, and 9 when tested in real-time RT-PCR specific for PRRSV-1, subtype 2 (Fig. 3). One of the VAC-T1T2 pigs did not respond until 5 DPC, where the first VAC-T1T2 pig became negative again. The mean Ct-value were comparable among the four groups, but vaccinated animals had in general higher Ct values compared to the unvaccinated control pigs especially at the later sampling days (Fig. 3).

PRRSV was detected in nasal swabs in all groups at DPCs 3–9 and the numbers of positive pigs were equal to or higher in the vaccinated groups compared to the unvaccinated controls (Table 2).

### 3.5. Challenge induced limited macroscopic changes

All pigs had increased size of Lnn Bronchiales, apart from 8 of the 17 VAC-T2 pigs (five challenged with PRRSV-1, subtype 1; one challenged with PRRSV-2 and one challenged with PRRSV-1, subtype 2) and two of the 15 VAC-T1 pigs (challenged with PRRSV-1, subtype 1). Following challenged with PRRSV-2, three

NON-VAC pigs had intestinal pneumonia and one NON-VAC pig showed signs of bronchopneumonia.

### 3.6. Vaccination had limited effect on PRRSV load in the lungs after challenge

One out of five pigs vaccinated and challenged with PRRSV-1, subtype 1, two out of five pigs vaccinated and challenge with PRRSV-2, three out of six pigs vaccinated with both vaccines and challenged with PRRSV-1, subtype 2 and four out of six pigs vaccinated with both vaccines and challenged with PRRSV-2 were negative for PRRSV in lungs when tested by real-time RT-PCR. All remaining pigs were positive for PRRSV in the lungs, including lung samples collected from affected areas. Vaccinated pigs had levels of virus in the lungs comparable to the control pigs when the load in the predetermined lung samples were analyzed, excluding the extra samples taken from affected areas (Fig. 4).

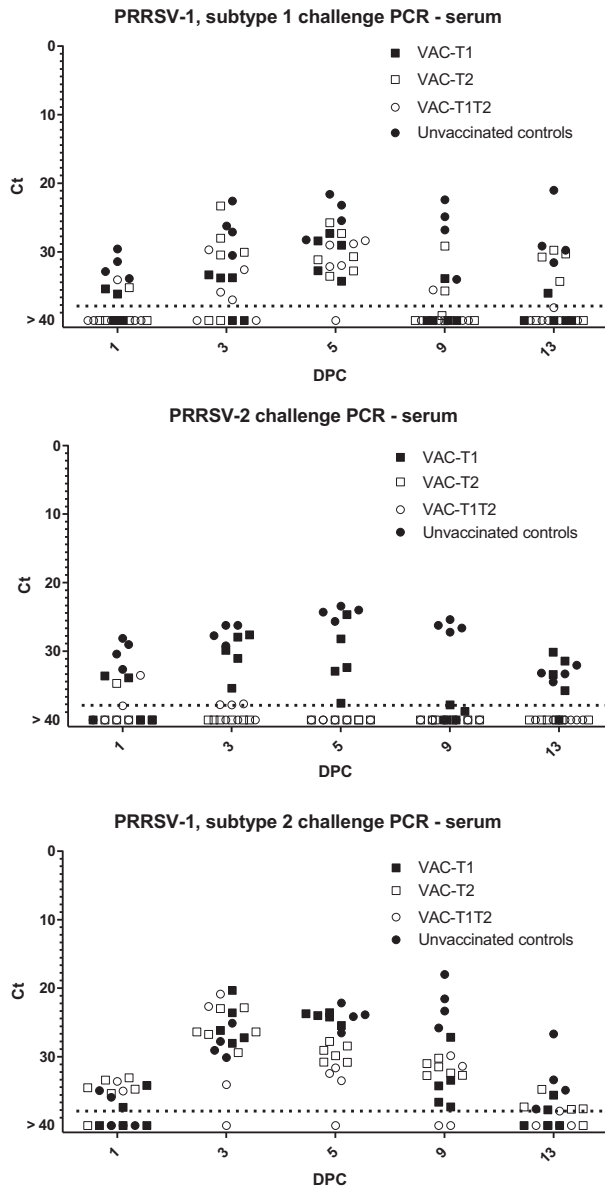
### 3.7. Sequencing of the challenge isolates

Sequencing of the ORF5 of the challenge strains, revealed a high level of identity (98.7% nt homology) between the PRRSV-2 challenge strain and the PRRSV-2 vaccine strain (Table 3). In contrast, the diversity between the PRRSV-2 vaccine strain and the PRRSV-1, subtype 1 and PRRSV-1, subtype 2 challenge strains were approximately 60%. The PRRSV-1 vaccine strain were 92, 82 and 62% identical to the PRRSV-1, subtype 1, PRRSV-1, subtype 2 and the PRRSV-2 challenge strains, respectively (Table 3).

Sequence analysis of ORF5 of the virus isolated from the lungs of one pig from each of the challenge groups were in all cases identical to the challenge strain (data not shown).

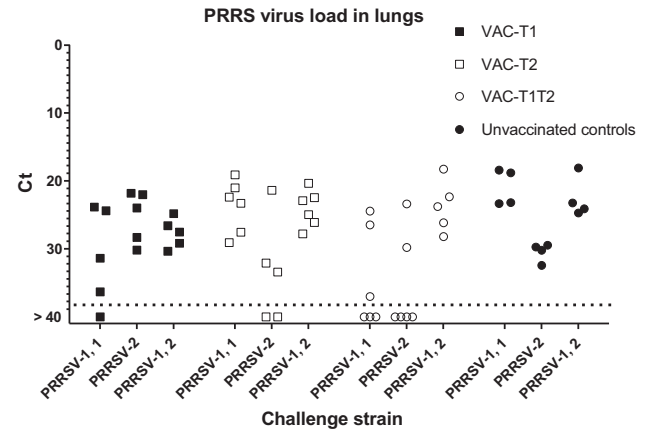
## 4. Discussion

The present study assessed the safety and efficacy of simultaneous administration of modified live vaccines containing attenuated PRRSV-1 and PRRSV-2 viruses, respectively. None of the vaccinated animals experienced any adverse effect following vaccination and comparable levels of virus were seen following the two vaccination strategies. Furthermore, no clear differences in responses were seen after challenge between animals vaccinated with one or two vaccines. Taken together, these results suggested that the safety and efficacy of two PRRSV MLV vaccines given simultaneously are similar to the safety and efficacy of the vaccines given alone.



**Fig. 3.** Viral load expressed as Ct values in serum after challenge with PRRSV-1, subtype 1 (top), PRRSV-2 (center) or PRRSV-1, subtype 2 (bottom). Groups of pigs were either unvaccinated or vaccinated with MLV against PRRSV-1 (VAC-T1), PRRSV-2 (VAC-T2) or both (VAC-T1T2) 63 days prior to challenge. The serum samples were tested with real-time RT-PCR assays specific for the challenge strain. Samples with Ct values above 40 were considered negative. DPC = days post challenge.

Following vaccination, the only statistical difference encountered was that the number of PRRSV-2 serum positive pigs overall were higher in the group vaccinated with PRRSV-2 compared to the group vaccinated with both vaccines ( $p = .007$ ), whereas the serum virus concentration was similar. The reason for this difference in response is not clear since the immune system is normally able to mount responses to several pathogens simultaneously. Furthermore, there was no clear difference between pigs vaccinated with PRRSV-1 and pigs vaccinated with both vaccines in the response to challenge with PRRSV-2. There are very few published studies on simultaneous vaccination with different PRRSV vaccines. Park and co-workers found that simultaneous vaccination with two vaccines against PRRSV-1 and PRRSV-2 and single vaccination were equally effective in reducing PRRSV viremia and lung lesions following PRRSV-1 challenge whereas simultaneous vaccination had



**Fig. 4.** Viral load expressed as Ct values in the lungs after challenge with PRRSV-1, subtype 1, PRRSV-2 or PRRSV-1, subtype 2. Groups of pigs were either unvaccinated or vaccinated with MLV against PRRSV-1 (VAC-T1), PRRSV-2 (VAC-T2) or both (VAC-T1T2) 63 days prior to challenge. The serum samples were tested with real-time RT-PCR assays specific for the challenge strain. Samples with Ct values above 40 were considered negative.

**Table 3**

Level of identity (nucleotide identity expressed as % identity in ORF5) among the vaccine and challenge strains used in the study.

| Vaccine           | Challenge strain | Type/subtype       | Identity (% nt) |
|-------------------|------------------|--------------------|-----------------|
| Porcilis®PRRS VET | 18794            | PRRSV-1, subtype 1 | 91.6            |
| Porcilis®PRRS VET | ILI6             | PRRSV-1, subtype 2 | 81.7            |
| Porcilis®PRRS VET | 19407b           | PRRSV-2            | 62.3            |
| Ingelvac®PRRS VET | 18794            | PRRSV-1, subtype 1 | 61.2            |
| Ingelvac®PRRS VET | ILI6             | PRRSV-1, subtype 2 | 60.7            |
| Ingelvac®PRRS VET | 19407b           | PRRSV-2            | 98.7            |

limited effect on viremia and lung lesions following challenge with PRRSV-2 [9]. This finding is in contrast to our findings and another study by the same group that showed protection against the same challenge strain in single vaccinated pigs [15]. The differences in outcome between the studies may be that Park et al. used another PRRSV-2 vaccine (Fostera PRRSV), a different challenge strain and the duration between vaccination and challenge was 35 days compared to the 62 days used in the present study. Furthermore, a more recent study showed that vaccination of boars with vaccines against PRRSV-1 and PRRSV-2 significantly reduced seminal shedding following challenge with the same PRRSV-2 strain [16].

Virus could be detected in serum of several pigs at DPC 42–56, and four (two VAC-T1 and two VAC-T2) pigs tested positive up to 62 days after vaccination. Previous studies have estimated the length of viremia after PRRSV vaccination with MLV vaccines to be 29 days on average (range 10–42 days) [12,17–20], but PRRSV viral RNA has been detected in tonsil scrapings up to 90 days post vaccination [20]. The different studies used different methods to investigate the presence of virus in serum, which may account for the differences seen. The sensitivity of different RT-PCR assays varies considerably and is dependent on a wide range of factors. One crucial determinant of sensitivity is the level of identity between the primer and probe sequences and the isolate to be tested [11]. In order to optimize the sensitivity of the virus detection assays employed, we chose to test for virus by real-time RT-PCR assays that were specifically validated and optimized to recognize the vaccine and challenge strains used in the present study. Therefore, optimized sensitivity of the assays employed may explain the long duration of viremia observed in our study compared to the majority of the previous studies. It cannot be ruled out that there was mutations in the probe and primer sites during



the replication in the pigs that would impair the sensitivity of the PCR, however, there was no indications that this happened in the present study. Another limitation of the study was that we stopped testing of samples after the first day they became negative excluded the possibility to detect a rebound of viremia – i.e. an animal that become positive after being tested negative. The duration of nasal excretion of PRRSV was in line with the previous studies quoted above (7–14 DPC). There is probably a limited risk that these weakly positive animals would transmit the virus horizontally to naïve animals [21], but the persistence of vaccine virus for two-three months after vaccination emphasize the importance of keeping vaccinated animals isolated from naïve animals for a sustained time period prior to mingling. Furthermore, vertical transmission from vaccinated boars for an extended time period after vaccination cannot be ruled out since the presence of virus in serum and semen is correlated [22].

One pig vaccinated with both vaccines tested positive very late (at DPV 56) and most likely this pig either had a delayed response to the vaccination or did not respond to vaccination at all and was infected by litter mates excreting vaccine virus.

Three different PRRSV strains were used as challenge virus. In West-European countries, circulating PRRSV-1, subtype 1 viruses can be divided into two main clusters with one of the clusters being highly similar to the PRRSV-1 reference strain LV (>95% identity in ORF 5), while some of the isolates from the other cluster differ more than 10% from the LV strain [7]. In the present study, the PRRSV-1, subtype 1 strain 18794 was used as challenge strain. This strain is an older Danish field isolate which share only 91.6% nt identity in ORF5 with the PRRSV-1 vaccine strain and 81.7% identity with the PRRSV-1, subtype 2 challenge strain ILI6. The strain ILI6 is a virus isolated in Russia in 2009 and belong to PRRSV-1, subtype 2 [23]. This subtype has to our knowledge never been detected in Western Europe [7].

Apart from sporadic detection of more diverse viruses in Hungary [24], all known European PRRSV-2 field viruses belongs to clade 5.1 and share high degree of identity to the PRRSV-2 vaccine strain VR2332 [8]. The Danish field strain used as challenge strain in the present study share 98.7% identity to the VR2332 strain. The term “homologous” and “heterologous” PRRSV challenge are often used to describe the degree of identity between PRRSV strains also in vaccine trials, but the terms have not been uniformly defined. In the present study it is reasonable to define the Type 2 challenge strain as homologous to the PRRSV-2 vaccine strain and both PRRSV-1 challenge strains to be heterologous to both vaccine strains. Therefore the efficacy of the two vaccines cannot be directly compared.

Few clinical signs were seen after challenge and only in the pigs challenged with PRRSV-1 subtype 2. The pronounced CRP responses seen on several sampling days, however, showed that all the pigs indeed responded to the challenge, and the responses were in accordance with previous results on the CRP response in pigs after PRRSV challenge [14]. The post challenge CRP responses and the clinical signs were more pronounced in vaccinated pigs, and in particular in pigs vaccinated with both vaccines compared to control pigs. The number of pigs included in each challenge group was relatively low and combined with the rather marked differences between the responses of pigs in the same group on the CRP measurements it was not possible to make clear conclusions on this, but these finding may indicate that the clinical signs as well as subclinical reactions (CRP acute phase response) encountered in response to PRRSV infection may be immune mediated [25].

In general, it is difficult to reproduce severe clinical signs in experimental trials with European field isolates of PRRSV especially in older pigs [5]. Instead, the magnitude and duration of virus in serum are often used as a correlate of protection in vaccination

trials. Pigs vaccinated against PRRSV-1 developed viremia shortly after challenge with PRRSV-1 subtype 1, however, the pigs cleared the virus from serum earlier than the other groups indicating partial protection by the vaccine in response to the heterologous PRRSV-1, subtype 1 virus challenge. There was no clear difference between single and dual vaccinated pigs in the response to challenge with PRRSV-1 virus. Some studies have found similar incomplete effect of vaccination following challenge of PRRSV-1 vaccinated pigs with heterologous PRRSV-1 strains [17,26], while others have found significant reduction in viremia following PRRSV-1 vaccination in a quasi-natural experimental model [27] and clinical protection in the field despite the field strain was only 85% identical to the vaccine strain in ORF 5 [19]. In the present study, vaccination against PRRSV-1 did not protect against challenge with Type 2 virus which are in accordance with the majority of previous studies [26,28–30]. The challenge in the present study was performed approximately two months after vaccination which is later than in most vaccine trials. The late challenge time was chosen because the recommended quarantine lengths for Danish gilts are minimum 8 weeks and we wanted to investigate if vaccine virus was present in serum and nasal swabs for a prolonged time after dual vaccination against PRRSV-1/PRRSV-2 compared to single vaccination. The apparent lack of protection seen for some of the groups may have been affected by this, but in a clinical setting the vaccines should be able to protect animals after challenge two months after vaccination. The limitation of using such a late challenge time was, however, that it was not possible to evaluate if the onset of immunity has been altered by vaccination with both vaccines.

PRRSV-2 vaccinated pigs were almost completely protected from viremia following challenge with the homologous PRRSV-2 strain which is in accordance with previous studies with this vaccine [5,31–33]. In contrast, vaccination with the PRRSV-2 vaccine had limited effect on viremia following challenge with PRRSV-1 which also is in accordance with the outcome of most of the previous comparable studies with this vaccine [5,26,34].

The challenge strain ILI6 belongs to PRRSV-1, subtype 2. PRRSV-1, subtype 2 and subtype 3 viruses have only been detected east of Poland and are more than 20% different from the PRRSV-1 subtype 1 strains circulating elsewhere [23]. Only few studies have been performed with these diverse East-European strains, but a series of studies have revealed that at least some subtype 3 strains are more virulent than typical subtype 1 strains [35–38]. Recently, we have shown that some subtype 2 strains seem to be more virulent than subtype 1 strains [10] and in both the previous and the present study ILI6 induced more severe clinical signs and more pronounced viremia than the PRRSV-1, subtype 1 strains. Nevertheless, the clinical signs were much less severe than seen following challenge with the two subtype 3 strains SU-1 and Lena [35,36]. Vaccination with PRRSV-1 and/or PRRSV-2 MLV vaccines had minimal reducing effect on the viral load in serum and lungs of ILI6 challenged pigs. Similarly, studies have shown that previous infection or vaccination with PRRSV-1 subtype 1 strains provided only partial protection against clinical signs and viremia following challenge with the subtype 3 strain Lena [39–41]. More studies are needed to investigate if the existing commercial vaccines will provide sufficient protection in the field against these diverse subtype 2–3 viruses.

Some of the pigs showed a biphasic trend in virus load in serum in that they became positive at DPC 3–6, negative at DPC 9 and then tested positive again at DPC 13. The retest of the samples confirmed the initial results. To our knowledge, this has not previously been described in connection to PRRSV challenge and it is interestingly that this trend only happened in vaccinated pigs challenged with a heterologous strain of PRRSV. Furthermore, there was a tendency that nasal shedding was more pronounced in vaccinated

animals challenged with a heterologous strain – especially in single vaccinated pigs challenge with ILI6. The underlining mechanisms behind these observations remain speculative, but antibody-dependent enhancement (ADE) has been shown to play a role in the pathogenesis of PRRSV infection in pigs [42], thus more studies on this is warranted.

## 5. Conclusion

None of the animals experienced any adverse effect following single or simultaneous vaccination with two PRRSV MLV vaccines and the viral load and duration of viremia were comparable between the two groups. Furthermore, no differences in responses of single and dual vaccinated animals were seen after homologous and heterologous challenge. In conclusion, the safety and efficacy of PRRSV MLV vaccines given simultaneously were similar to the safety and efficacy of the vaccines given alone. This information is very useful for decision making in swine herds situated in areas where both PRRSV-1 and PRRSV-2 are circulating.

## Competing interests

None.

## Acknowledgements

This work was partly funded by the Boehringer Ingelheim European PRRS Research Award 2014.

Heidi Gertz Andersen is thanked for expert assistance with CRP determinations.

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